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(54) Title: NOVEL FUNGICIDAL PROPERTIES OF METABOLITES, CULTURE BROTH, STILBENE DERIVATIVES AND INDOLE DERIVATIVES PRODUCED BY THE BACTERIA *XENORHABDUS* AND *PHOTORHABDUS* SPP.

(57) Abstract

The present invention relates to the use of microbes of the genera *Xenorhabdus* and *Photorhabdus* and the refined or raw metabolites of same from culture media for use as a fungicide for agricultural, horticultural, veterinary or human use. The present invention further relates to the use of dihydroxyalkyl-substituted stilbene derivatives of the formula: 3,5-dihydroxy-4-R-trans-stilbene; where R is an alkyl group of carbon length 1 to 6 with either a straight chain or branched configuration, such as 3,5-dihydroxy-4-isopropyl-trans-stilbene or 3,5-dihydroxy-4-ethyl-trans-stilbene, as fungicides which have protectant, preventative and eradicant capabilities. The invention also relates to the use of 3-substituted indole derivatives of the formulas: 3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole, 3-(2'-acetoxy-4'-methyl-3'-oxopentyl)-indole, 3-(2'-hydroxy-4'-methyl-3'-oxohexyl)-indole, and 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole and their stereoisomer as fungicides which have protectant, preventative and eradicant capabilities.

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NOVEL FUNGICIDAL PROPERTIES OF METABOLITES, CULTURE BROTH, STILBENE DERIVATIVES AND INDOLE DERIVATIVES PRODUCED BY THE BACTERIA *XENORHABDUS* and *PHOTORHABDUS* spp.

FIELD OF THE INVENTION

This invention relates to fungus disease control employing biological agents especially for the benefit of agriculture, food storage, garden, ornamental plants, trees and wood products, humans, animals, and other materials and organisms mitigated by fungal organisms. In particular, it relates to the preventative, protective and eradicative treatment of fungal diseases of all types through the use of biological agents and the raw and partially refined metabolites found from the bacterial genera *Xenorhabdus* and *Photorhabdus*.

This invention futher relates to employing the stilbene derivatives of the form 3,5-dihydroxy-4-R-*trans*-stilbene; where R is an alkyl group of carbon length 1 to 6 with either a straight chain or branched configuration, such as 3,5-dihydroxy-4-*iso*propyl-*trans*-stilbene or 3,5-dihydroxy-4-ethyl-*trans*-stilbene. The latter compounds are produced by the bacterial symbiont *Photorhabdus luminescens* and other *Xenorhabdus* species, or are produced by chemical methods, and are useful for fungus disease

control for the benefit of agriculture, food storage, garden, ornamental plants, trees and wood products, humans, animals, and other materials and organisms mitigated by fungal organisms. In particular, it relates to the preventative, protective and eradicative treatment of fungal diseases of all types through the use of these stilbene derivatives.

This invention also relates to employing the indole derivatives 3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole, 3-(2'-acetoxy-4'-methyl-3'-oxopentyl)-indole, 3-(2'-hydroxy-4'-methyl-3'-oxohexyl)-indole, and 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole, and their stereoisomers, produced either by the bacterial symbiont *Xenorhabdus bovienii* and other *Xenorhabdus* and *Photorhabdus* species and or by chemical method for fungus disease control for the benefit of agriculture, food storage, garden, ornamental plants, trees and wood products, humans, animals, and other materials and organisms mitigated by fungal organisms. In particular, it relates to the to the preventative, protective and eradicative treatment of fungal diseases of all types through the use of these indole derivatives (3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole, 3-(2'-acetoxy-4'-methyl-3'-oxopentyl)-indole, 3-(2'-hydroxy-4'-methyl-3'-oxohexyl)-indole, and 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole) produced by *Xenorhabdus bovienii* and other *Xenorhabdus* and *Photorhabdus* species.

BACKGROUND TO THE INVENTION

Fungi are eucaryotic micro-organisms characterized by parasitic and saprophytic habits. Many derive nutritional benefit through invasion of hosts and deprivation of essential materials. Fungi may affect plants and animals by causing reduced vigor,

inhibition of growth, physical damage and deformation, reduced fecundity or even host death.

Over 200 fungi are recognized as human pathogens among the 100,000 or so species known. About 20 generate systemic infections, another 20 are regularly found as cutaneous infections, and a dozen or more are identified with severe, localized subcutaneous diseases. In addition, there is a long list of opportunistic fungi that cause diseases in debilitated patients, and these, like *Candida albicans*, can be endogenous to humans. Debilitated persons with acquired immunodeficiency syndrome (AIDS) are found to be very susceptible to secondary infections of fungi, especially after antibiotic treatment for bacterial infections. *Candida albicans*, *C. krusei*, *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Coccidioides immitis* are the more common secondary infections following AIDS. With the predicted worldwide increase in AIDS cases in the 1990's, prevention and treatment of these secondary infections by effective fungicides becomes increasingly important, especially as the infective fungi develop resistance to antifungal agents. Currently, dermal infections by fungi are treated topically with chemical agents (e.g., Castederm, Desenex, Vioform), antifungal synthetic agents (e.g., Tolnaftate, Tinactin, Loprox), antifungal imidazole derivatives (e.g., Miconazole, Micatin, Lotrimin), antifungal allylamine derivatives (e.g., Naftifine, Naftin) or antifungal and steroid combinations (e.g., Clotrimazole + Betamethasone, Lotrisone). A new class of antimycotics, the triazoles, show some promise as broad-spectrum treatments for fungus infection and they can be given orally. They are effective against some of the yeasts, such as *Candida* sp, and against aspergillosis.

Whereas species of virus, bacteria and nematodes that cause plant disease are numbered in the hundreds, plant-pathogenic fungi number in the thousands. Fungi probably cause more than 100,000 diseases in green plants alone. Protection of agricultural crops, stored foods, gardens, ornamental plants, trees and wood products, fish and animals from fungal disease requires applications of materials to:

(a) prevent attack from germinating reproductive stages such as spores, conidia, and conidospores at times when the host is particularly sensitive (e.g. new seedlings), or when climatic conditions or other stresses are particularly conducive to new fungal growth (e.g. high humidity);

(b) protect hosts from invasion by the actively growing fungal tissues, such as mycelial mats or hyphal extensions; and

(c) eradicate growing fungal disease organisms after the host has been attacked, thus mitigating damage and preserving the host from further harm and possible destruction.

Conventional, petroleum-based fungicidal pesticides are limited in their scope and abilities to fulfill these requirements for all potential pest situations. Some products are limited by the types of disease organisms they kill, while others are limited by their effectiveness as preventatives or eradicants. Many have very short-lived effects in the environment because of degradation by ultra-violet light, chemical effects (e.g., ozonolysis), adsorption onto soil particles and, in many cases, through degradation by other microbes, especially bacteria which occur naturally in the soils. Synthetic pesticides also have detrimental effects on the environment and its inhabitants. Regulatory pressures are reducing the future usefulness of such

conventional pesticides. An important attribute of a useful fungicide is its differential toxicity to the fungi and the host. Thus, fungicides for treatment of plant disease must have little or no phytotoxicity. Low water solubility of the substance may minimize the phytotoxic components to the host plant, but allow it to remain available to affect pathogenic fungi. Materials used in treatments also must have low toxicity to animals. For a fungicide to be useful it must not be carcinogenic, teratogenic or cause sub-toxic effects.

Many natural interactions exist between the microscopic flora and fauna of soils. Attempts have been made to select beneficial soil microbes antagonistic to fungal pathogens, thus providing biological control systems for the benefit of crop production and food storage. Unfortunately, due to the complexity of the inter-relationships of large numbers of these soil organisms, it has proved frustrating to isolate single microbes of beneficial action. It has been postulated that this poor result is because these soil inhabitants, through existence in a common environment and through continued interaction, have faced similar evolutionary pressures, and through their mutual interactions, evolved to a mutual, albeit antagonistic, existence. It is further a disadvantage of other known biological control agents that conventional pesticides have proved to be more effective and easier to handle. Thus, very few useful fungicidal agents of natural origin have been discovered. Those biological control agents whose effect is of benefit to man are of limited scope (i.e. the eradication of grey molds on stored foods) and have yet to be generally commercialized.

Bacteria of the genera *Xenorhabdus* and *Photorhabdus* (Enterobacteriacea) were first discovered inside microscopic worms called nematodes. The nematodes act as vectors which carry the bacteria into insects. In particular, bacteria of these genera are carried within these entomopathogenic nematodes which inject them into insect hosts, thereby killing the host. This nematode-bacteria complex was recognized as a biological insecticide and subsequently commercialized.

The infective juvenile stage of the nematode carries the living bacteria in a non-reproducing stasis inside its gut while the nematode travels freely through the soils seeking insect hosts. As such, the bacteria are protected from many outside biotic and abiotic influences. The bacteria have thereby avoided co-evolution with other soil inhabitants. The nematode, upon finding a host, enters the insect through oral, anal and other orifices and then penetrates into the body cavity.

Once in the body cavity, the nematode releases factors which activate the insect's immune system. This response kills any unprotected microbes present inside the insect, including those accidentally introduced by the invading nematode. After the insect response subsides, the nematode expels the *Xenorhabdus* or *Photorhabdus* spp. bacteria from its gut, into the insect haemocoel (internal body space) and the bacteria grow and multiply slowly. The bacteria cause septicemia and produce metabolites which kill the insect. Such metabolites are known to be antibiotics which kill other bacteria that may have invaded the insect host during the interim. Once the insect host is dead, the nematode feeds on the multiplying *Xenorhabdus* bacteria and then enters

a reproductive state. The host fills with infective juvenile nematodes, each of which carries live *Xenorhabdus* or *Photorhabdus* cells. The insect cadaver bursts, thus releasing tens of thousands of questing nematodes ready to seek new insect hosts. Thus, it was presumed that the insecticidal and anti-bacterial activities were the key roles of the genera *Xenorhabdus* and *Photorhabdus*.

Different *Xenorhabdus* species and a *Photorhabdus* species are characteristically associated in monoculture with different species of entomopathogenic nematodes.

The cell-free culture broths of *Photorhabdus luminescens* (and other closely related species), a bacterial symbiont carried by nematodes of the genus *Heterorhabditis* and other *Xenorhabdus* species carried by nematodes of the genus *Steinernema*, were found to be active against many fungi of agricultural and medicinal importance (Chen, G. and Webster, J.M., U.S. Patent Application No. 08/097,968, Filed 07/27/93 and Chen, G., G. Dunphy and J.M. Webster, "Antifungal Activity of Two *Xenorhabdus* Species and *Photorhabdus luminescens*, Bacteria Associated with the Nematodes *Steinernema* Species and *Heterorhabditis megidis*." *Biological Control* 4:157 (1994)). In order to understand the nature of this bioactivity and to develop more bioactive products, the isolation and identification of the bioactive components from this bacteria were pursued.

Although there is a limited number of publications on these genera of bacteria, it has been recognized that active, anti-microbial substances are produced by *Xenorhabdus* species and the closely-related *Photorhabdus* species. Some of these specific

compounds have been isolated, identified and their structures elucidated. However, the importance of these specific purified metabolites as extremely potent fungicides or fungistatic agents has heretofore been unrecognized.

The usefulness of the *Xenorhabdus* and *Photorhabdus* species, their unrefined cells, culture media and raw or partially refined metabolic products have been undiscovered until now because of certain aspects newly recognized, that are the subjects of this invention. It is believed that it has not heretofore been shown that operable aspects of *Xenorhabdus* or *Photorhabdus* exist for use in its raw or partially refined or cultured form as fungicide or fungistatic agents. Furthermore, it has been unrecognized until now that, through the complex life cycle of the subject bacteria of the genus *Xenorhabdus* and *Photorhabdus*, the most potent antifungal and fungistatic activities occur only at and after a key point in the life cycle of the bacteria; within the dead or dying insect.

The usefulness of both the stilbene derivative compounds and the indole derivative compounds found in these bacteria have been undiscovered until now because of certain aspects newly recognized, that are the subjects of this invention. Prior art references have not shown use of these specific compounds or any operable aspects as fungicide or fungistatic agents.

In artificial culture, it has been found that both *Xenorhabdus* and *Photorhabdus* bacteria undergo a phase change from their primary form. The primary form is found in the nematode or within infested insects. These primary forms produce lecithinase, absorb dyes,

contain paracrystalline inclusions and produce antibiotics and pigments, whereas secondary forms produce little or no antibiotics and pigments. The precise cause of the phase change is unknown, but it can be induced under certain culture conditions, and can occur spontaneously in artificial culture.

Antibiotic production has been reported from artificial culture of phase one of *Xenorhabdus nematophilus*, *X. bovienii* and *Photorhabdus luminescens*. The activity spectrum of the antibiotics varies with the species and strain of bacteria. Four types of antibiotics produced by the bacterial species have been described: (1) indole derivatives from *X. bovienii* (Paul *et al.*, "Antibiotics in Microbial Ecology: Isolation and Structure Assignment of Several New Antibacterial Compounds from the Insect-Symbiotic Bacteria *Xenorhabdus* spp." *J. Chem. Ecol.* Vol. 7, pp. 589-597 (1981)), (2) stilbene derivatives from *P. luminescens* (Ibid; Richardson *et al.*, "Identification of an anthraquinone pigment and a hydroxystilbene antibiotic from *Xenorhabdus* [*Photorhabdus*]" *App. Environ. Microbiol.* Vol. 54, pp. 1602-1605 (1988)); (3) the organically-soluble dithiolopyrrolones, xenorhabdins, for *Xenorhabdus* sp. strain Q, *X. bovienii* and strains of *X. nematophilus* (McInerney *et al.* "Biologically Active Metabolites from *Xenorhabdus* spp., Part 1. Dithiolopyrrolone derivatives with antibiotic activity" *J. Nat. Prod.* Vol. 54, pp. 774-784 (1991)); and (4) the water-soluble benzopyran derivatives, xenocoumacins, for *Xenorhabdus* strain Q and the All strain of *X. nematophilus* (McInerney *et al.* "Biologically Active Metabolites from *Xenorhabdus* spp., Part 2. Benzopyran-1-one derivatives with gastroprotective activity" *J. Nat. Prod.* Vol. 54, pp. 785-795 (1991)).

Akhurst, "Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the Families Heterorhabditidae and Steinernematidae" *J. Gen. Microbiol.* Vol. 128, pp. 3061 - 3065 (1982) demonstrated anti-bacterial inhibition by killed *Xenorhabdus* colonies grown on artificial media. He also showed inhibition of three species of yeast, including the human pathogen, *Candida albicans*, with extracts containing low-molecular-weight factors from *Xenorhabdus*, and stated there was no activity from factors of molecular weight greater than 14,000 when *Xenorhabdus* spp. were artificially cultured. Akhurst, however, did not examine the bacterial response within the insect, thereby failing to cause the bacteria to produce its most potent response.

Paul *et al.* (1981) examined nine strains of *Xenorhabdus* (and *P. luminescens*) in artificial culture and identified active chemicals. They identified two dihydroxy stilbene derivatives from *X. luminescens* (Strain Hb) by mass spectrometry and NMR as 3,5-dihydroxy-4-*iso*propyl-*trans*-stilbene and 3,5-dihydroxy-4-ethyl-*trans*-stilbene. They also identified four 3-substituted indoles from *X. nematophilus* (Strain R) by mass spectrometry and NMR as 3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole, 3-(2'-acetoxy-4'-methyl-3'-oxopentyl)-indole, 3-(2'-hydroxy-4'-methyl-3'-oxohexyl)-indole, and 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole, but they did not examine the bacteria *in vivo*. Paul *et al.* did not test for fungicidal activities of these stilbene derivatives.

McInerney *et al.*, J. Nat. Prod., *54*, 774-784 (1991); *54*, 785-795 (1991); and their corresponding patents Rhodes *et al.* "Process for Making Xenorhabdin

Antibiotics", U.S. Patent 4,672,130 (June 9, 1987) and Gregson et al." Xenocoumacins", U.S. Patent 4,837,222 (June 6, 1989), respectively, elucidated the structures of various low-molecular-weight active chemicals from *Xenorhabdus*. While they demonstrate an anti-bacterial activity for xenorhabdins and xenocoumacins their technique evaluated the fungistatic rather than the fungicidal properties. They postulated potential "antifungal" properties on the basis of chemical similarity with another group of compounds, without demonstration of fungicidal activity. Furthermore, they used only refined materials of low molecular weight without recognition of the potent complex of low and high molecular weight factors which forms during the natural growth cycle within host insects. There is no teaching on the use of raw bacteria and raw or partially refined bacterial products as fungistatic and fungicidal materials.

Richardson *et al.* (1988) identified one dihydroxy stilbene from *X. luminescens* (Strain HK) by NMR and mass spectrometry as 3,5-dihydroxy-4-*iso*propyl-stilbene.

Sundar, L. and F.N. Chang "The role of Guanosine-3'-5'-Bis-Pyrophosphate in Mediating Antimicrobial Activity of the Antibiotic 3,5-Dihydroxy-4-Ethyl-*trans*-Stilbene" *Antimicrobial Agents and Chemotherapy* 36:2645-2651 (1992) examined the mode of action of 3,5-dihydroxy-4-ethyl-*trans*-stilbene as an antimicrobial. However, Sundar and Chang and the references therein revealed no prior knowledge of the antimycotic nature of 3,5-dihydroxy-4-ethyl-*trans*-stilbene or of 3,5-dihydroxy-4-*iso*propyl-*trans*-stilbene.

The stilbenes found in nature which are fungicidal are referred to as phytoalexins, and are produced by host plants in response to fungal infections. These compounds are found in various unrelated plants, such as peanut, grape, sugar cane and pine. The compounds of the instant invention arise from a unique source in nature: bacteria. Their specific 4-substitution is also unique in nature.

Hart, "Role of Phytostilbenes in Decay and Disease Resistance" *Ann. Rev. Phytopathol.* 19:437-458 (1981) and references therein recognize that 14-carbon phenolic stilbenes have (page 437) "long been known for their antifungal properties," but noted that chemical analogues of phytoalexins are not necessarily anti-fungal.

Sundar, L. and F.N. Chang "Antimicrobial Activity and Biosynthesis of Indole Antibiotics Produced by *Xenorhabdus nematophilus*." *J. Gen. Microbiol.* 139: 3139-3148 (1993), (p. 3139): "investigated the mechanism of action and physiology of production of the indole derivative antibiotics." They report additional effectiveness of the derivatives against both Gram-positive and Gram-negative bacteria by "causing a severe inhibition of RNA synthesis, accompanied by a less severe effect on protein synthesis." They include no fungal organisms in their activity survey.

A group of 3-substituted indole compounds contain an ester at the 3-indole position, such ester extended with an alkyl, benzyl or indolyl carbonyl group (Kikuchi *et al.* "Indole Derivatives." U.S. Patent 5,124,324 (Jun. 23, 1992). These compounds are claimed as selective antagonists of 5-HT_3 (hydroxytryptamine) receptors, which are useful for

the treatment of psychotic disorders, neurotic diseases, gastric stasis symptoms, gastrointestinal disorders, nausea and vomiting.

A *Photorhabdus* sp. (previous known as *X. luminescens*) has been found in association with human wounds (Farmer *et al.* 1989). Although they are similar to *Xenorhabdus* and *Photorhabdus* found in host nematodes, the bacteria associated with human wounds have a different range of adaptation; as indicated by their optimal temperature being the same as that for warm-blooded animals. Colepicolo *et al.* (1989) characterized these bacteria according to the effects of ionic strength, temperature, oxygen, and iron on the bacteria's growth and development. In addition, the culture medium after growth of this species exhibited antibiotic activity.

Cochrum *et al.* (1990), Meighen (1991) and Xi *et al.* (1992) cloned the luminescence genes of *Photorhabdus luminescens* and found that the enzymes were structurally very similar to luminescence enzymes of other terrestrial bacteria and marine bacteria. Colepicolo *et al.* (1992) found that, unlike several species of marine bioluminescent bacteria, human wound *Xenorhabdus* could grow in an atmosphere of pure oxygen, indicating a significant functional difference from other luminescent bacteria at physiological levels.

SUMMARY OF THE INVENTION

It is therefore an objective of this invention to produce naturally-based fungicides which have protectant, preventative and eradicant capabilities. It is further an objective to produce low cost

material, through organic synthesis methods or by the use of bacteria (alive and killed), and raw, unrefined or partially refined, or purified metabolites from within the bacteria and found in its culture media, separately and in combination, that is an effective fungicide.

Furthermore, it is an objective to produce fungicides with broad-spectrum capabilities to kill fungal diseases of many classes and in many forms, such as reproductive, vegetative or resting stages. An additional objective is to produce such a fungicide which is easy to formulate and apply using conventional pesticide-application equipment and methods.

A further objective of this invention to produce naturally-based fungicides which have protectant, preventative and eradicant capabilities in controlling fungi which are human pathogens.

An additional objective of this invention to utilize stilbene derivatives of 3,5-dihydroxy-4-substituted-*trans*-stilbene where the substituent is an alkyl group of carbon length 1 to 6 with either a straight chain or branched configuration, such as 3,5-dihydroxy-4-*iso*propyl-*trans*-stilbene or 3,5-dihydroxy-4-ethyl-*trans*-stilbene as fungicides which have protectant, preventative and eradicant capabilities.

Another objective of this invention to utilize indole derivatives of the formulas: 3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole, 3-(2'-acetoxy-4'-methyl-3'-oxopentyl)-indole, 3-(2'-hydroxy-4'-methyl-3'-oxohexyl)-indole, and 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole and their stereoisomers, as

fungicides which have protectant, preventative and eradicant capabilities.

Still further objects and advantages shall become apparent from consideration of the ensuing descriptions and preferred embodiments.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

It has been discovered that *Xenorhabdus* and *Photorhabdus* spp. (Enterobacteriaceae) and their raw or partially refined metabolites, as well as specific metabolites, have a beneficial effect by destroying many pathogenic fungi.

Bioassays of the antifungal activity of *Xenorhabdus* spp. and *Photorhabdus* sp., and bioassays of the antifungal activity of stilbene derivatives from *Photorhabdus luminescens* and indole derivatives from *Xenorhabdus* spp., were done by the methods based on the standard procedure of the American Society of Phytopathology for testing chemical fungicides. Sources and depositories of *Xenorhabdus* and *Photorhabdus* species and strains are noted in Akhurst and Boemare "A numerical taxonomic study of the genus *Xenorhabdus* (Enterobacteriaceae) and proposed elevation of the subspecies of *X. nematophilus* to species" *J. Gen. Microbiol.* Vol 134, pp. 1835-1845 (1988). Putz *et al.* "Development and application of oligonucleotide probes for molecular identification of *Xenorhabdus* species" *Appl. Environ. Microbiol.* Vol. 56, pp. 181-186 (1990) notes additional sources and depositories, including the American Type Culture Collection, Rockville, MD. They identify and separate 35 strains of *Xenorhabdus* into one of five species:

Xenorhabdus beddingii

Xenorhabdus bovienii
Xenorhabdus poinarii
Xenorhabdus luminescens
Xenorhabdus nematophilus

Further characterization of *Xenorhabdus* is provided by Akhurst and Boemare (1988), where both phases and 21 strains were examined for 240 characters. *Xenorhabdus luminescens* has recently been placed into a separate genus and is now classified as *Photorhabdus luminescens*.

Candidate fungal pathogens used in bioassays are readily available from many sources, including the American Type Culture Collection, Rockville, MD.

The species of *Xenorhabdus* and their nematode symbionts used in all studies were collected and maintained in culture. Last instar larvae of the greater wax moth, *Galleria mellonella*, were infected with infective juvenile (IJ) nematodes, carrying the respective *Xenorhabdus* spp. at a rate of 25 IJs/larvae. After 24 to 48 hours (h) the dead insect larvae were surface disinfected by dipping them into 95% ethanol and igniting them. The cadavers were aseptically opened with sterile forceps, haemolymph (fluid plasma and blood cells) from the haemocoel was streaked onto an NBTA medium (nutrient agar supplemented with 0.025 grams (g) of bromothymol blue and 0.04 g of 2,3,5-triphenyltetrazolium chloride per liter) and incubated in the dark at room temperature. The resulting primary form *Xenorhabdus* spp. of each isolate was maintained and subcultured at 14 day intervals. For consistency, glycerinated (17%/nutrient broth) stocks of bacteria frozen at -18°C were frequently used as starting materials for cultures. Inocula of the primary form were prepared

by adding one loop-full of the culture to 50 milliliters (ml) of tryptic soy broth (TSB) in a 100 ml Erlenmeyer flasks. Cultures were shaken at 120 rpm on an Eberbach gyrorotary shaker for 24 hours at 25°C. Bacterial fermentation was initiated by adding 100 ml of the bacterial culture (OD_{600} at ≈2.0) to 900 ml of TSB in a 2,000 ml flask. The flask is incubated in the dark at 25°C on an Eberbach gyrorotary shaker. After 96 hours, the culture is centrifuged (12,000 xg, 20 minutes, 4°C) to separate the bacterial cells. The cell-free material is then extracted with ethyl acetate. The organic phase is then separated into fractions, from which the active stilbene or indole derivatives can be isolated.

The active fungicidal ingredients of the composition of the present invention include a culture medium containing nonviable bacteria, the culture medium from which the bacterial cells have been removed, the nonviable bacteria *per se*, or filtrates of the culture medium. A preferred filtrate of the culture medium will be a filtrate from which compounds of molecular weight of lower than about 750 gm/mole have been excluded. A particularly preferred filtrate has compounds of molecular weight lower than about 500 gm/mole excluded therefrom. Heat treatment of the culture medium or bacteria is also an alternative. Typically, treatments above about 50°C up to about 121°C at 15 psi may be utilized from periods of about 10 to 30 minutes.

The relative simplicity of the individual organic molecules of the present instance lend themselves to organic synthetic methods, in addition to microbial production methods. Such standard synthetic processes are described in the literature, such as, for the stilbene derivatives, Bachelor *et al.*

"Synthesis of Pinosylvin and Related Heartwood Stilbenes" *Can J. Chem.* 48:1554 (1970) and Krow *et al.* "Synthesis of Antibiotic Stilbenes Using Organomanganese Arene Complexes" *J. Org. Chem.* 57:4040 (1992) and the references therein.

The fungicidal compositions may be formed using one of the active ingredients in an inert carrier. If formulated as a solid, the ingredient(s) may be mixed with typical carriers such as Fuller's earth, kaolin clays, silicas or other wettable inorganic diluents. Free-flowing dust formulations may also be utilized by combining the dry active ingredient with finely divided solids such as talc, kieselguhr, pyrophyllite, clays, diatomaceous earth and the like.

The powders may also be applied as a suspension or solution, depending on the solubility in the liquid carrier. Pressurized sprays, typically aerosols with the active ingredient dispersed in a low-boiling dispersant solvent carrier, may be used.

Percentages of weight may vary according to the manner in which the composition is to be applied, and formulation used. In general, the active ingredient will comprise 0.005% to 95% of the active ingredient by weight in the fungicidal composition. The fungicidal composition may be applied with other ingredients, including growth regulators, insecticides, fertilizers, etc.

Formulation of the active ingredients to assist applicability, ease handling, maintain chemical stability and increase effectiveness may require addition of various materials. Solvents may be chosen on the basis of affecting the solubility of the active ingredient, fire hazard and flash point,

emulsifiability, specific gravity and economic considerations. Adjuvants may be added to enhance the active ingredients, and can include surfactants which are anionic, cationic or nonionic. Stabilizers and antifreeze compounds will prolong storage. Additionally, synergists, stickers, spreaders and deodorant compounds can be added to improve the handling characteristics of the commercial formulation.

Alternatively, the active ingredient can be combined with an inert carrier, such as calcium carbonate, and formed into a pill or other consumable delivery device, including controlled-release devices intended to deliver metered doses of the active ingredient.

The fungicidal compositions are useful against fungi which are harmful to agricultural crops, animals and desirable botanical species, such as ornamental and flowering plants. Generally, application doses will be in the range of 100 to 1,000 ppm of active ingredient to inert carrier. However, dosages will vary based on the particular fungi, host and environment.

EXAMPLE 1

Effect of *Xenorhabdus*-killed insect cadavers on soil microbes.

Galleria larvae killed by either injection of 5 µl of *Xenorhabdus* (e.g., *X. nematophilus*, ATCC No. 19061, American Type Culture Collection, Rockville, MD.) bacterial suspension (5.0×10^6 bacteria/ml) or by traumatic insult through repeated needle punctures were placed for comparison into soils collected from natural sites. After 8 days, the *Xenorhabdus*-treated replicates contained significantly fewer soil

microbes than the untreated samples. The test was originally intended to measure effects on soil bacteria, however it was noted also that the fungal populations were unexpectedly affected. This led to further investigations of these results.

EXAMPLE 2

Timing of production of active metabolites by *Xenorhabdus* spp. under natural conditions.

To assess the natural production of active materials by *Xenorhabdus*, insect larvae were artificially infested and antibiotic production measured. *Galleria* larvae were injected with 10 microliters (μl) of phosphate-buffered saline containing either (i) *X. nematophilus* (e.g. ATCC No. 19061), (ii) surface-disinfected IJ nematodes or (iii) nematodes containing no bacterial symbiont. Larvae were incubated at 27 degrees Celsius (°C) and extracted for antibiotics at death and every 24 h thereafter until 144 h post-injection.

Antibiotics were extracted by homogenizing the insects in distilled water at a ratio of 1 ml/larva and the suspension stirred on a magnetic plate for 20 minutes. The resulting mixtures were centrifuged (6000 g., 20 minutes, 24°C) to remove insect tissue and fat droplets. The supernatants were adjusted to pH 7.0, centrifuged (12,000 g; 20 minutes, 4°C), filter sterilized (0.22μ) and stored at 4°C. Fifty ml of filtrate was pipetted into each well (Ø = 0.5 cm), cut on TSA (10 ml in each 9-cm plate) freshly inoculated with *Bacillus subtilis* spore (6×10^9 CFU). The diameter of inhibition zone size was determined after 24 hours of incubation at 25°C.

Antibiotic activity was expressed as the radius of the annular clearing around the antibiotic disk.

Antibiotic activity was detected after the insect was killed. TABLE 1 shows that *Xenorhabdus* alone, and *Xenorhabdus* with the nematode symbiont produced highest antibiotic titres after 24 hours, while the bacteria-free treatment produced no antibiotics. Antibiotic levels dropped when the nematode was present, but were sustained when only the bacteria were introduced. This study showed that a key factor activated the *Xenorhabdus* under natural conditions.

TABLE 1: Antibiotic Activity of *Xenorhabdus* under natural conditions in the insect host (diameter inhibition zone, cm ± standard error)

TREATMENT	TIME AFTER INJECTION (hours)						
	0	24	48	72	96	120	144
X. nematophilus	0	0	2.10±0.06	2.35±0.06	2.40±0.06	2.40±0.06	2.50±0.06
Nematode with *Xenorhabdus*	0	0	2.23±0.10	2.17±0.10	2.50±0.10	2.33±0.09	2.20±0.15
Nematode without bacteria	0	0	0	0	0	0	0

EXAMPLE 3

Fungicidal activity of *Xenorhabdus* filtrates on mycelium growth of *Pythium* spp.

Xenorhabdus spp. (e.g., *X. nematophilus*, ATCC No. 19061, ATCC No. 39497, ATCC No. 53200, *X. bovienii*, ATCC No. 35271) and *Photorhabdus* sp. (e.g. *P.* (Xenorhabdus) *luminescens*, ATCC No. 29999) were cultured separately in agitated broth medium incubated at 25°C for 5 days. The spent culture

media were then neutralized with 6 Normal (N) hydrochloric acid (HCl), centrifuged at 11,000 xg for 20 m to remove bacterial cells, precipitated, and the filtrates were collected and filter sterilized with an effective pore size of 0.2 micron (μ) before bioassay.

The filtrates of *X. nematophilus* and *X. bovienii*, prepared as above without filtration, were further adjusted to pH 5.8 with HCl, centrifuged at 11,000 xg for 10 m filter sterilized and lypholized (lypholization has no effect on the efficacy of the filtrates) to yield a concentrated filtrate powder. The powder was added to potato dextrose agar (PDA) at 49°C and mixed thoroughly to give the final filtrate concentration required for bioassay.

To determine the fungicidal or fungistatic property of the filtrate, fungal discs of the test fungus were placed in the center of Petri dishes containing the filtrate-supplemented PDA. Those discs which had mycelium growth totally inhibited by *Xenorhabdus* were removed from the filtrate-supplemented plates and placed into new filtrate-free PDA plates, incubated at 24°C. The growth of those discs in the filtrate-free plates was further investigated 4 days after the removal and incubation. Those filtrate concentrations from which no hyphal growth occurred in any one of six replicate discs were defined as fungicidal and those from which hyphal growth occurred in any one of the replicated discs were defined as fungistatic.

Results: *Xenorhabdus* spp. filtrates were fungicidal to *Pythium splendens*, *P. sulcatum*, *P. sylvaticum* and *P. ultimum* when they were supplemented in PDA at a concentration of 30%.

EXAMPLE 4

Fungicidal activity f *Xenorhabdus* filtrates on macroconidal and conidial germinations f *Fusarium solani*, *F. oxysporum* and *Botrytis cinerea*.

The methods of Example 3 above were used for this study. The macroconidia of *F. solani* and *F. oxysporum* and the conidia of *B. cinerea* were prepared by growing the fungi on PDA at 24°C and these plates were flooded with sterile water when the conidia were ready to be harvested. The conidia were washed twice with sterile distilled water, centrifuged, re-suspended in 15% sterile potato dextrose broth, diluted to give concentrations of $6x10^3$ macroconidia/ml, $7x10^3$ macroconidia/ml, and $5x10^3$ conidia/ml for *F. solani*, *F. oxysporum* and *B. cinerea* respectively, and bio-assayed for germination.

The bioassay of the conidial germination was performed in depression slides incubated in a humid chamber. Conidial germination after incubation was determined under the microscope based on three counts of 100 conidia for each replicate. In the case of no or low germination, at the time of observation, the slides were re-incubated and re-examined after further incubation.

Results: The filtrates of *Xenorhabdus* spp. were fungicidal to the germination of macroconidia of *Fusarium solani*, *F. oxysporum* and conidia of *Botrytis cinerea*.

EXAMPLE 5

Heat-stable and larg molecular properties and their fungal activity on ma r conidial germination of *Fusarium solani*, *F. oxysporum*, and the conidial germination of *Botrytis cinerea* and the mycelial growth of *Pythium ultimum* .

The methods of Example 3 were used in this study. The culture filtrates of the *X. nematophilus* were either heated in a water bath at 60°, 100°C or autoclaved at 121°C and 15 pounds per square inch (psi) for 20 m or were dialyzed in dialysis tubing of molecular weight cut-off (MWC) of 8,000 to 9,000 and 12,000 to 14,000 overnight in running distilled water, thus removing materials of lower molecular weights. The dialysates were concentrated using a Rotavapour-R Büchi at room temperature, then reconstituted with distilled water. The heat-treated and the dialyzed filtrates were then adjusted to pH 5.8 or 7, centrifuged at 11,000 xg at room temperature for 10 m to remove the precipitates, filter sterilized (0.22μ) and bio-assayed for their effect on the growth of *Pythium ultimum* and on the macroconidial germination of *F. solani*, *F. oxysporum* and the conidial germination of *B. cinerea*.

Results: Heat-treated and the high molecular weight dialyzed filtrates were fungicidal to the macroconidial germination of *Fusarium solani*, *F. oxysporum*, and conidial germination of *Botrytis cinerea* and the mycelial growth of *Pythium ultimum*.

EXAMPLE 6

Broad spectrum fungicidal activity of *Xenorhabdus* and *Photorhabdus* species.

The fungi to be tested were maintained on potato dextrose agar (PDA) in the dark at room temperature. Fifty microliters of the TSB culture of each *Xenorhabdus* or *Photorhabdus* isolate were transferred into the center of a 9 cm Petri dish containing 10 ml TSB plus 1.5% agar, and incubated in the dark at 24°C for 4 days. Each plate was subsequently inoculated in a triangular fashion with the test fungal mycelial plug (0.5 cm) cut from cultures freshly grown on PDA plates. Each inoculation was replicated three times using three plates. Control plates were inoculated with TSB minus *Xenorhabdus*. The growth of the fungi was observed and recorded over a period of 4 to 80 days after inoculation, depending on the species of fungus. The longer incubation times were required for slow-growing fungi.

The *Xenorhabdus* and *Photorhabdus* isolates used are from the laboratory of Dr. J.M. Webster (Biological Sciences Department, Simon Fraser University, Burnaby, B.C. Canada, V5A 1S6), as follows:

D1 *X. nematophilus* D1 from *Steinernema carpocapsae* nematode

DS *X. nematophilus* D1 secondary form as above

BC1 *X. nematophilus* BC1 from *Steinernema carpocapsae* nematode

A2 *X. bovienii* A2 from *S. feltiae*

A3 *X. bovienii* A3 from *S. feltiae*

NC *X. bovienii* NC from *S. feltiae*

BC2 *X. bovienii* BC2 from *S. feltiae*

C9 *P. luminescens* from *Heterorhabdits megidis*

TABLE 2 shows the results. Antimycotic substance(s) inhibited fungal growth to an extent that varied with the fungal species and the bacterial isolate. The growth of all fungi except for the mycorrhizal fungus *Suillus pseudobrevipes* was inhibited to some extent by one or more of the *Xenorhabdus* or *Photorhabdus* isolates. Seven species of plant pathogenic fungi had their growth completely inhibited by the primary form of all of the *Xenorhabdus* and *Photorhabdus* (*Botrytis cinerea, Ceratocystis ulmi, C. cryocoetidis, Mucor piriformis, Pythium coloratum, P. ultimum* and *Trichoderma pseudokoningii*. Considerable resistance was demonstrated by *Aspergillus niger, Cephaloascus fragrans, Beauveria bassiana* and *Metarhizium anisopliae* (both insect pathogens) and by the mycorrhizal fungi *Oidiodendron griseum* and *S. pseudobrevipes*. It is notable that the xenocoumacin 1 of McInerney was shown to affect *Aspergillus niger*. The mycorrhizal fungi are beneficial species existing in commensual relationships with plants. Their resistance to treatment would thus be useful. Surprisingly, antifungal activity was seen even in the second phase of *Xenorhabdus* (i.e., SFU Strain DS), where the current teachings state no anti-biotic activity should be present.

In addition to the fungi in TABLE 2, the activity of the compounds of the present invention also encompasses:

Malassezia furfur, Trichosporon beigelii, Piedraia hortae, Microsporum sp., *Trichophyton* sp., *Epidermophyton* sp., *Candida albicans, Fonsecaea pedrosoi, Pseudallescheria boydii, Madurella mycetomtis, Basidiobolus ranarum, Conidiobolus coronatus, Rhinosporidium seeberi, Loboa loboi, Sporothrix schenchii, Histoplasma capsulatum,*

Blastomyces dermatitidis, *Paracoccidioides brasiliensis*, *occidioides immitis*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Mucor* sp, *Absidia* sp., *Rhizopus* sp., *Rhizomurcor* sp., *Candida albicans*, *Pseudallesscheria boydii*, *Wangiella dermatitidis*, *Phialophora* sp., *Paelcilomyces* sp., *Beauveria* sp., *Scopulariopsis* sp., *Prototheca* sp., *Schizophyllum commune* and *Coprinus* sp.

TABLE 2: Spectrum of Antimycotic Activity of Species and Strains of *Xenorhabdus* spp. and *Photorhabdus luminescens*

Fungal Species	*Activity of Xenorhabdus Species*						
	D1	*DS*	*BC1*	*BC2*	*A3*	*A2*	*C9*
Alternaria sp. A	++	0	++	++	++	++	+
Alternaria sp. B	++	0	++	++	++	++	+
Aspergillus niger	-	0	+	+	-	+	-
Beauveria bassiana	-	-	+	+	+	+	-
Botrytis cinerea	+++	++	+++	+++	+++	+++	+++
Cephaloascus fragrans	+	0	+	+	+	+	-
Ceratocystis ulmi	+++	0	+++	+++	+++	+++	+++
C. dryocoetidis	+++	0	+++	+++	+++	+++	+++
Fusarium oxysporum	++	++	++	++	++	++	++
F. solani	++	++	++	++	++	++	++
Geotrichum candidum	++	+	++	++	++	++	++
Gleosporium perannans	++	0	++	++	++	++	-
Metarhizium anisopliae	-	-	+	-	-	-	-
Monilina fructicola	++	0	++	++	++	+	-
Mucor piriformis	+++	++	+++	+++	+++	+++	+++
Oidiodendron griseum	-	0	+	-	+	-	-
Ophiostoma piceae	++	0	++	++	++	++	++
Pythium coloratum	+++	+++	+++	+++	+++	+++	+++
P. ultimum	+++	++	+++	+++	+++	+++	+++
Penicillium expansum	++	0	++	++	++	+	+
P. notatum	+++	0	+++	++	++	+	++
Penicillium sp.	+++	0	+++	++	++	+++	+
Rhizoctonia cerealis	+++	++	+++	+++	+++	+++	++
R. solani	+++	++	+++	+++	+++	+++	++
Rhizopus stonifer	++	0	++	++	++	++	++
Sclerotinia minor	++	0	+++	+++	++	++	++
Suillus pseudobrevipes	-	0	-	-	-	-	-
Thielaviopsis basicola	++	0	+++	+++	++	+++	++
Trichoderma pseudokingii	+++	0	+++	+++	+++	+++	+++
Venturia inaequalis	+	0	+	+	+	+	+
Verticillium albo-atrum	++	0	+++	++	++	++	++
V. dahliae	+++	0	+++	++	+++	++	+++

KEY:
+++ clear inhibition zone that persisted for at least 1 week.
++ clear inhibition zone that was subsequently colonized by aerial hyphae or small clusters of hyphae after a few days.
\+ a zone of poor fungal growth surrounding the *Xenorhabdus* inoculum
\- no inhibition
0 not tested

EXAMPLE 7

Isolation of th active stilbene components From *Photorhabdus luminescens*

The cell-free culture broth of *P. luminescens* (SFU strain C9) was extracted by combining an equivalent volume of acetyl acetate in a separatory funnel and collecting the organic phase. The process was repeated three times. The organic-phase extracts were combined, dried over anhydrous sodium sulfate and filtered through glass wool to remove the inorganic materials. The liquid filtrate was evaporated by rotatory evaporator (Büchi) held under 30°C under a vacuum. The resulting concentrated crude material was then separated into different fractions with flush silica gel chromatography (silica gel 60 in 50 cm X 8 cm glass column) with first 30% ether in hexane, then 60% ether in hexane and finally 100% ether. The most active fraction was found to be present in the middle eluate, which upon concentration gave the active compound ST1.

EXAMPLE 8

Identification of the active stilbene components From *Photorhabdus*

^{1}HNMR spectrum of ST1 was recorded on a Bruker WM400 spectrometer in $CDCl_3$, using residual $CHCl_3$ (~7.25) as internal standard. The mass spectrum was obtained on a Hewlett-Packard 5985B GC/MS system operating at 70 eV using a direct probe. ^{13}CNMR datum was recorded on the same instrument in CD_3OD. The IR spectrum was recorded on a Perkin-Elmer S99B spectrometer. (Abbreviations used as follows: EI = Electron Impact, M^+ = Molecular Ion, s = singlet, t = triplet, q=quartet, J = coupling constant, Hz = Hertz, d =

doublet, hept = heptet, AB = a pair of protons separated by a small chemical shift).

EIMS: 256 (M^+ +2,3%), 255 (M^+ +1,8%), 254 (M^+ 38%), 240 (18%), 211 (4%), 239 (100%), 211 (4%), 205 (9%), 179 (5%), 178 (9%), 165 (12%), 149 (18%), 129 (12%), 125 (9%), 123 (9%), 111 (16%), 109 (12%), 105 (8%), 97 (23%), 95 (18%), 91 (11% 0), 85 (16%), 84 (8%), 83 (26%), 82 (11%), 81 (26%), 73 (9%), 71 (22%), 70 (10%), 69 (44%), 67 (11%), 57 (8%), 56 (8%), 55 (24%).

^{1}HNMR ($CDCl_3$): 7.47 (2H, d, J=7 Hz), 7.33 (3H, t, J=8 Hz), 6.94 (2H, AB, J= 16 Hz), 6.50 (2H, s), 4.73 (2H,s), 3.43 (1H, hept, J=7 Hz), 1.37 (6H, d, J=7 Hz).

^{13}CNMR (CD_3OD): 157.7 (s), 139.1 (s), 136.9 (s), 130.0 (d), 129.6 (d), 128.4 (d), 128.3 (d), 127.3 (d), 122.2 (s), 106.6 (d), 25.7 (d), 21.0 (q).

IR (KBr): 3548, 3427, 1610, 1579, 1570, 1449, 1428, 1348, 1348, 1278, 1238, 1436, 1069, 1015, 994, 968, 817, 751, 690, 628 cm^{-1}.

EXAMPLE 9

Bioassays of the active stilbene components From *Photorhabdus*.

Active compound 3,5-dihydroxy-4-*isopropyl*-*trans*-stilbene was dissolved in dimethylsulfoxide (DMSO), filter sterilized with a 0.22 μ filter and diluted into 2 mls of potato dextrose broth (PDB) in a 5 ml flask, resulting a final DMSO concentration of <0.2%(v/v). The active compound was serially diluted by twofold to produce culture media containing from 100 μg/ml to 0.1 μg/ml of active ingredient (i.e., 100, 50, 25, 12.5, 6.3, 3.2, 1.6, 0.8, 0.4, 0.2, and

0.1 μg/ml) in order to determine the minimum inhibitory concentration (MIC). Each dilution was replicated 3 times with both liquid media and agar plates. The two yeast test candidates (*Candida tropicales* and *Cryptococcus neoformans*) were grown on potato dextrose agar (PDA) for 24 hours at 25°C, then scraped from the plate by flooding the plate with 8% saline and diluted with the saline to make inocula containing 2.5 to 2.8 x 10^7 Colony Forming Units (CFU) per ml. *Aspergillus* spp. and *Botrytis cinerea* were grown for 7 days at 25°C before the conidia were harvested by flooding the plate with sterile, distilled water and diluted to make the final inocula of 2.5 to 3.0 x 10^6 conidia per ml. The inocula were then added to treated culture media. Replicates were incubated at 35°C (*B. cinerea* was incubated at 24°C) and the MIC visually determined after 24 hours (48 hours for *B. cinerea*). The MIC is defined as the lowest concentration of active compound which prevents the growth of the test organism within 24 hours.

RESULTS: It was found that similar effects were seen from both liquid and agar culture methods. TABLE 3 shows the MICs determined for the compound against each fungus organism.

TABLE 3: Minimum Inhibitory Concentrations (MIC) of chemical isolated from *Photorhabdus luminescens* on candidate fungal pathogens.

Organisms	MICs (μg/ml) 3,5-dihydroxy-4-*iso*propyl-*trans*-stilbene
Aspergillus fumigatus	12
A. flavus	25
Botrytis cinerea	25
Candida tropicales	12
Cryptococcus neoformans	12

EXAMPLE 10

Isolation of the active indole components From *Xenorhabdus*

The cell-free culture broth of *Xenorhabdus bovienii* (e.g., SFU Strain A2 or ATCC No. 35271) was extracted by combining an equivalent volume of ethyl acetate in a separatory funnel and collecting the organic phase. The process was repeated three times. The organic-phase extracts were combined, dried over anhydrous sodium sulfate and filtered through glass wool to remove the inorganic materials. The liquid filtrate was evaporated by flash evaporator (Büchi) held under 35°C under a vacuum. The resulting concentrated crude material was then separated into different fractions with flash silica gel chromatography (silica gel 60 in 50 cm X 8 cm glass column) with 50% ether in hexane. Pure compound one (ID1) was eluted first, followed by a mixture of ID1 and compound two (ID2), then pure ID2, a mixture of ID2 and compound three (ID3), followed by pure ID3, a

mixture of ID3 and compound four (ID4), and finally pure ID4.

EXAMPLE 11

Identification of the active indole components From *Xenorhabdus*

NMR spectra of each of ID1, ID2, ID3 and ID4 were recorded on a Bruker WM400 spectrometer in $CDCl_3$, using residual $CHCl_3$ (~7.25) as internal standard. Low resolution mass spectra were obtained on a Hewlett-Packard 5985B GC/MS system operating at 70 eV using a direct probe. High resolution MS spectra were recorded on a Kratos MS80 instrument. IR spectra were recorded as neat film on NaCl using a Perkin-Elmer S99B spectrometer. (Abbreviations used as follows: EI = Electron Impact, M+ = Molecular Ion, t = triplet, J = coupling constant, Hz = Hertz, d = doublet, m = multiplet, sext = sextet, dd = doublet doublet, q = quartet, bs = broad singlet, hept = heptet).

ID1: identified as 3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole; mass spectrum, EI, m/z (relative intensity): 287 (M+, 10), 227 (M+, -60, 10), 170 (47), 130 (100), 103 (10), 84 (25), 77 (20), 57 (35); 1H NMR, δ: 0.86(3H, t, J=7 Hz), 0.98 (3H, d, J=6.5 Hz), 1.39 (1H, m), 1.73 (1 H, m), 2.07 (3H, s), 2.63 (1H, sext, J=7 Hz), 3.18 (1H, dd, J=7 Hz, J=15 Hz), 3.30 (1H, dd, J=4.5 Hz, J=15 Hz), 5.46 (1H, q, J=4.5 Hz, J=8 Hz), 7.05 (1H, d, J=2.2 Hz), 7.15 (1H, t, J=7.5 Hz), 7.21 (1H, t, J=7.5 Hz), 7.37 (1H, d, J=7.5Hz), 7.64 (1H, d, J=7.5 Hz), 8.06 (1H, bs, NH).

ID2, identified as 3-(2'-acetoxy-4'-methyl-3'-oxopentyl)-indole: mass spectrum, EI, m/z (relative intensity): 223 (M+, 25), 213 (M+ -60, 15), 170 (60),

130 (100), 103 (6), 84 (12), 77 (8); 1H NMR, δ: 0.99 (3H, d, J=6 Hz), 1.09 (3H, d, J=6 Hz), 2.07 (3H, s, $COCH_3$), 2.72 (1H, hept, J=7 Hz), 3.19 (1H, dd, J=7 Hz, J=15 Hz), 3.27 (1H, dd, J=4.5 Hz, J=15 Hz), 5.46 (1H, dd, J=4.5 Hz, J=8 Hz), 7.05 (1H, d, J=2.2 Hz), 7.15 (1H, t, J=7.5 Hz). 7.21 (1H, t, J=7 5 Hz). 7.37 (1H, d, J=7.5 Hz), 7.64 (1H, d, J=7.5 Hz), 8.06 (1H, bs, NH).

ID3 identified as 3-(2'-hydroxy-4'-methyl-3'-oxohexyl)-indole: mass spectrum, EI, m/z (relative intensity): 245 (M+, 10), 130 (100), 92 (5), 77 (3); 57 (2); mass spectrum, CI, isobutane: 246 (M+1, 100), 130 (55); IR (film), cm^{-1}: 3410, 2967, 2932, 1705, 1457, 742; 1HNMR δ: 0.875 (3H, t, J=7 Hz), 0.99 (3H, d, J=6.5 Hz), 1.42 (1H, m), 1.65 (1H, m), 2.77 (1H, sext, J= 6.5 Hz), 3.04 (1H, dd, J=7 Hz, J=15 Hz), 3.32 (1H, dd, J=4.5 Hz, J=15 Hz), 3.52 (1H, d, J= 5.5Hz, OH), 4.6 (1H, m), 7.12 (1H, d, J= 2.3Hz), 7.15 (1H, t, J=7.5 Hz), 7.2 (1H, t, J=7.5 Hz), 7.36 (1H, d, J=7.5 Hz), 7.63 (1H, d, J=7.5 Hz), 8.06 (1H, bs, NH). 13C NMR, δ: 11.39, 14.78, 27.07, 29.83, 43.11, 76.23, 110.89, 111.21, 118.64, 119.54, 122.15, 122.81, 127.43, 136.13, 215.82.
High resolution mass spectrum calculated for $C_{15}H_{19}NO_2$: 245.1416, found: 245.1416. Long-range ^1H-^{13}C correlation spectroscopic result: the carbon with chemical shift at 110.89 ppm was coupled with the proton with chemical shift at 3.04 ppm (1H, dd, J=7 Hz, J=15 Hz).

ID4 identified as 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole: mass spectrum, EI, m/z (relative intensity): 231 (M+, 15), 130 (100), 103 (5), 77 (10). 1H NMR, δ: 1.02 (3H, d, J=6.5 Hz), 1.13 (3H, d, J=7 Hz), 2.88 (1H, hept, J=7 Hz), 3.06 (1H, dd, J=7 Hz, J=15 Hz), 3 31 (1H, dd, J=4.5 Hz, J=15 Hz),

3.45 (1H, d, J=5.5 Hz, OH), 4 67 (1H, m), 7.11 (1H, d, J=2.3 Hz), 7.14 (1H, t, J=7.5 Hz), 7.2 (1H, t, J=7.5 Hz), 7.36 (1H, d, J=7.5 Hz), 7.63 (1H, d, J=7.5 Hz), 8.05 (1H, bs, NH)

EXAMPLE 12

Bioassays of the active indole components From *Xenorhabdus*

Active compounds ID1, ID2, ID3 and ID4 were dissolved in dimethylsulfoxide (DMSO), filter sterilized with a 0.22 micron filter and diluted into 2 ml of potato dextrose broth (PDB) in a 5 ml flask, resulting a final DMSO concentration of <0.2% (v/v). The active compounds were serially diluted twofold to produce culture media containing from 100 μg/ml to 0.1 μg/ml of active ingredient (i.e., 100, 50, 25, 12.5, 6.3, 3.2, 1.6, 0.8, 0.4, 0.2, and 0.1 μg/ml) in order to determine the minimum inhibitory concentration (MIC). Each dilution was replicated three times with both liquid media and agar plates. The two yeast test candidates (*Candida tropicales* and *Cryptococcus neoformans*) were grown on potato dextrose agar (PDA) for 24 hours at 25°C, then scraped from the plate by flooding the plate with 0.8% saline and diluted with the saline to make inocula containing 2.5 to 2.8 x 10^7 Colony Forming Units (CFU) per ml. *Aspergillus* spp. and *Botrytus cinerea* were grown on PDA for 7 days at 25°C before the conidia were harvested by flooding the plate with sterile, distilled water and diluted to make the final inocula of 2.5 to 3.0 x 10^6 conidia per ml. The inocula were then added to treated culture media. Replicates were incubated at 35°C (*B. cinerea* was incubated at 24°C) and the MIC visually determined after 24 hours (48 hours for *B. cinerea*). The MIC is defined as the lowest concentration of active compound which prevents the

growth of the test organism at the above conditions.

RESULTS: It was found that similar effects were seen from both liquid and agar culture methods. TABLE 4 shows the MICs determined for each compound against each fungus.

TABLE 4: Minimum Inhibitory Concentrations (MIC) of chemicals isolated from *Xenorhabdus bovienii* on candidate fungal pathogens.

Organisms	MICs (µg/ml)			
	ID1	ID2	ID3	ID4
Aspergillus fumigatus	>100	>100	>100	>100
A. flavus	>100	>100	>100	>100
Botrytis cinerea	>100	>100	12.5	12.5
Candida tropicales	>100	>100	>100	>100
Cryptococcus neoformans	50	25	>100	>100

ID1: 3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole
ID2: 3-(2'-acetoxy-4'-methyl-3'-oxopentyl)-indole
ID3: 3-(2'-hydroxy-4'-methyl-3'-oxohexyl)-indole
ID4: 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole

EXAMPLE 13

Effect of *Xenorhabdus nematophilus* on the opportunistic human fungal pathogen, *Candida albicans*.

Xenorhabdus nematophilus (e.g, SFU Strain D1 or ATCC 19061) was cultured in broth medium at 25°C on an oscillating shaker. After 5 days of growth, the spent culture was neutralized with 6 normal hydrochloric acid and centrifuged at 11,000xg for 20 minutes at 4°C to remove bacteria cells. The filtrates were sterilized by filtration with a 0.2 micropore filter (Millipore, Bedford, MA). An agar

diffusion bioassay was routinely used to test for antibody activity. Bacterial agar medium was autoclaved for 15 minutes and then held in a water bath at 50°C for one hour. Ten milliliters of medium were poured into a 10 cm petri dish. Once the petri dishes cooled, they were stored at room temperature until utilized in tests. The bacteria and fungi were cultured in their appropriate broth media, shaken at 80 rpm at 25°C overnight and 100 µl of bacteria or fungi was then spread evenly over the surface of medium of each bioassay petri dish. The dishes were placed into a laminar air-flow hood for 20-30 minutes to dry. Two to four wells (0.5 cm in diameter) were cut into the agar of each inoculated petri dish, using a alcohol-flame sterilized cork borer. Fifty microliters of test solution were added into each well, the dishes were covered with lids and placed at 25°C. Following 24 hours of incubation, the diameter of the inhibition zone was measured with hand-held calipers.

RESULTS: In all replicates that included the *Candida albicans* on plates containing the spent culture medium of *Xenorhabdus nematophilus* a significant clear area was present, indicating that growth of *Candida albicans* was inhibited by components in the medium.

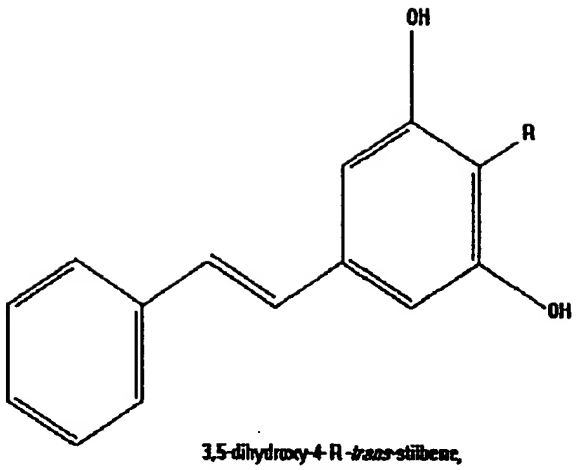
In conclusion, it is shown that potent antifungal properties of *Xenorhabdus* species and *Photorhabdus* species and their raw or partially purified metabolites affect a broad spectrum of fungal pathogens. The dialization treatment separated these effects from known, lower-molecular weight substances of specified molecular structure. The use of raw, unrefined culture media, partially refined culture

media, bacteria and bacterial extracts were found to be highly potent.

It was further shown that newly-discovered potent antifungal properties of *Xenorhabdus nematophilus* and its purified, raw or partially purified metabolites affect the growth of an opportunistic human pathogen, *Candida albicans*.

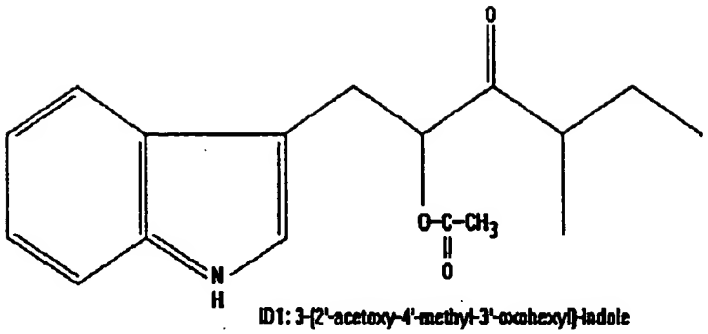
Furthermore, it is shown that the compound 3,5-dihydroxy-4-*iso*propyl-*trans*-stilbene shows potent antifungal properties, and that differential effects against fungal pathogens occur with each compound. Additionally, it is shown that these compounds 3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole, 3-(2'-acetoxy-4'-methyl-3'-oxopentyl)-indole, 3-(2'-hydroxy-4'-methyl-3'-oxohexyl)-indole, and 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole show potent antifungal properties, and that differential effects against fungal pathogens occur with each compound.

While our above description contains many specificities, these should not be construed as limitations on the scope of the inventions, but rather as examples of preferred embodiments. Accordingly, the scope of the invention should not be determined by the embodiments presented, but by the appended claims and their legal equivalents.

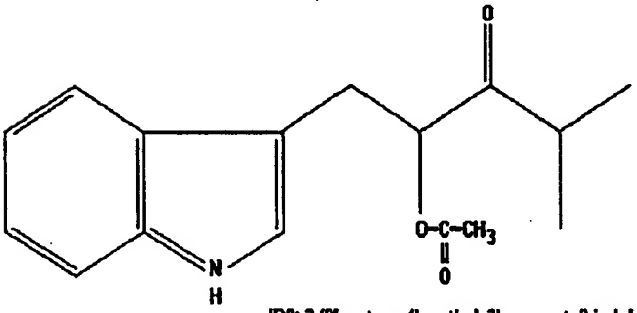


3,5-dihydroxy-4-R-*trans*-stilbene,

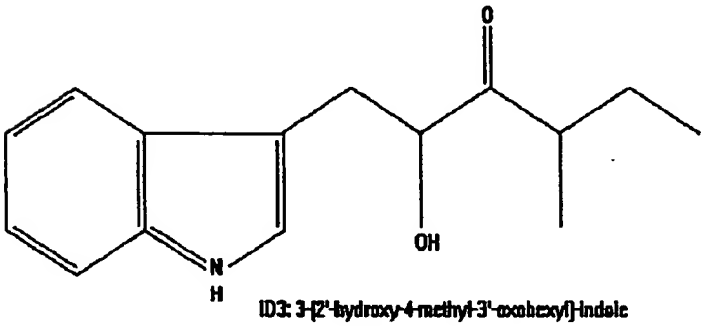
where R is an alkyl group of carbon length 1 to 6 with either a straight or branched chain configuration



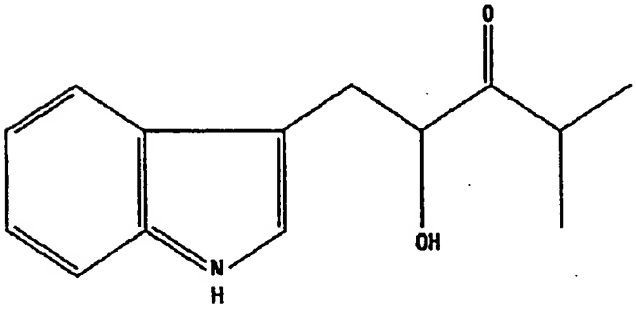
ID1: 3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole



ID2: 3-(2'-acetoxy-4'-methyl-3'-oxopentyl)-indole



ID3: 3-(2'-hydroxy-4-methyl-3'-oxohexyl)-indole



ID4: 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole

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WHAT IS CLAIMED IS:

1. A fungicidal composition comprising:

a fungicidally effective amount of culture medium containing bacteria, said bacteria selected from the group consisting of the genus *Xenorhabdus*, the genus *Photorhabdus*, and mixtures thereof; and

a biologically inert carrier.

2. A composition according to Claim 1 wherein said bacteria are nonviable.

3. A composition according to Claim 1 wherein said fungicidally effective amount of culture medium is sufficient to kill fungi on desirable agricultural crops and botanical species.

4. A composition according to Claim 1 wherein said bacteria is predominantly in its primary form.

5. A composition according to Claim 1 wherein said bacteria are in a lyophilized form.

6. A composition according to Claim 1 wherein said bacteria are in a spray-dried form.

7. A composition according to Claim 1 wherein said culture medium is heat-treated.

8. A composition according to Claim 1 wherein said culture medium is a filtrate, said filtrate having a lower molecular weight cutoff excluding molecules less than about 500 gm/mole.

9. A fungicidal composition, comprising:

a fungicidally effective amount of culture medium fr m which bacterial cells have been removed, said medium derived from growing bacteria selected from

the group consisting of the genus *Xenorhabdus*, the genus *Photorhabdus*, and mixtures thereof; and
a biologically inert carrier.

10. A composition according to Claim 9 wherein said fungicidally effective amount of culture medium is sufficient to kill fungi on desirable agricultural crops and botanical species.

11. A composition according to Claim 9 wherein said bacteria is predominantly in its primary form.

12. A composition according to Claim 9 wherein said culture medium is heat-treated.

13. A composition according to Claim 9 wherein said culture medium is a filtrate, said filtrate having a lower molecular weight cutoff excluding molecules less than about 500 gm/mole.

14. A fungicidal composition, comprising:
a fungicidally effective amount of nonviable bacteria, said bacteria selected from the group consisting of the genus *Xenorhabdus*, the genus *Photorhabdus*, and mixtures thereof; and
a biologically inert carrier.

15. A composition according to Claim 14 wherein:
said fungicidally effective amount of bacteria is sufficient to kill fungi on desirable agricultural crops and botanical species.

16. A composition according to Claim 14 wherein:
said bacteria is predominantly in its primary form.

17. A composition according to Claim 14 wherein said bacteria are in a lyophilized form.

18. A composition according to Claim 14 wherein said bacteria are in a spray-dried form.

19. A composition according to Claim 14 wherein said bacteria is heat-treated.

20. A composition according to Claim 14 wherein:
said bacteria is predominantly in its secondary form.

21. A process for producing a fungicidal composition, comprising:
infecting insect larvae with infective juvenile nematodes, said nematodes carrying bacteria species selected from the group consisting of the genus *Xenorhabdus*, the genus *Photorhabdus*, and mixtures thereof;
allowing said insect larvae to die as a result of said infection;
collecting said *Xenorhabdus* or *Photorhabdus* bacteria from cadavers of said insect larvae, when said bacteria exhibits its most potent fungicidal activity;
combining a fungicidally effective amount of said culture medium containing said bacteria with a biologically inert carrier.

22. A process according to Claim 21 further comprising the step of culturing said bacteria in culture medium after collecting same from said cadavers.

23. A process according to Claim 22 further comprising the steps of

obtaining filtrates of said culture medium excluding m lecules having a molecular weight of lower than about 500 gm/mole; and

using a fungicidally effective amount of said filtrate in place of said culture medium to combine with said biologically inert carrier.

24. A process according to Claim 22 further comprising the step of removing cells of said bacteria from said culture medium.

25. A method for the control of fungi, comprising:

contacting said fungi or their habitats with a fungicidally effective amount of a fungicidal composition according to any one of Claims 1 through 20.

26. A method according to Claim 25 wherein said fungi is selected form the group consisting essentially of *Pythium splendens, P. sulcatum, P. sylvaticum, P. ultimum, Botrytis cicerea, Ceratocystis ulmi, C. dryocoetidis, Mucor piriformis, Pythium coloratum, Trichoderma pseudokingii, Fusarium solani, F. oxysporum, Alternaria sp., Rhizoctonia cerealis, R. solani, Sclerotinia minor, Thielaviopsis basicola, Verticillium albo-atrum, V. dahliae, Geotrichum candidum, Gleosporium perannans, Monilina fructicola, Ophiostoma piceae, Penicillium expansum, P. notatum, Penicillium sp., Rhizopus stonifer* and *Venturia inaequalis*.

27. A composition according to Claim 1 wherein said fungicidally effective amount of culture medium is sufficient to kill fungi on humans.

28. A method according to Claim 25 wherein said fungi are selected from the group consisting essentially

of, *Malassezia furfur, Trichosporon beig lii, Piedraia hortae, Microsporum* sp., *Trichophyton* sp., *Epidermophyton* sp., *Candida albicans, Fonsecaea pedrosoi, Pseudallescheria boydii, Madurella mycetomtis, Basidiobolus ranarum, Conidiobolus coronatus, Rhinosporidium seeberi, Loboa loboi, Sporothrix schenchii, Histoplasma capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis, occidioides immitis, Cryptococcus neoformans, Aspergillus fumigatus, Mucor* sp, *Absidia* sp., *Rhizopus* sp., *Rhizomurcor* sp., *Candida albicans, Pseudallesscheria boydii, Wangiella dermatitidis, Phialophora* sp., *Paelcilomyces* sp., *Beauveria* sp., *Scopulariopsis* sp., *Prototheca* sp., *Schizophyllum commune* and *Coprinus* sp.

29. A fungicidal composition comprising:
a fungicidally effective amount of 4-substituted 3,5-dihydroxy-*trans*-stilbene; where the substituent is an alkyl group of carbon length 1 to 6 with a straight chain or branched configuration and
a biologically inert carrier.

30. A fungicidal composition according to Claim 29 comprising:
a fungicidally effective amount of 3,5-dihydroxy-4-*iso*propyl-*trans*-stilbene; and
a biologically inert carrier.

31. A fungicidal composition according to Claim 29 comprising:
a fungicidally effective amount of 3,5-dihydroxy-4-ethyl-*trans*-stilbene; and
a biologically inert carrier.

32. A composition according to Claims 29, 30, or 31, wherein said effective amount of active material is

sufficient to kill fungi on agricultural crops and botanical species.

33. A composition according to Claims 29, 30, or 31 wherein said effective amount of active material is sufficient to kill fungi on agricultural livestock or other animals, humans or fish.

34. A fungicidal composition comprising:
a fungicidally effective amount of 3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole; and
a biologically inert carrier.

35. A fungicidal composition comprising:
a fungicidally effective amount of 3-(2'-acetoxy-4'-methyl-3'-oxopentyl)-indole; and
a biologically inert carrier.

36. A fungicidal composition comprising:
a fungicidally effective amount of 3-(2'-hydroxy-4'-methyl-3'-oxohexyl)-indole; and
a biologically inert carrier.

37. A fungicidal composition comprising:
a fungicidally effective amount of 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole; and
a biologically inert carrier.

38. A fungicidal composition comprising:
a fungicidally effective amount of a mixture of at least two of the components 3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole, 3-(2'-acetoxy-4'-methyl-3'-oxopentyl)-indole, 3-(2'-hydroxy-4'-methyl-3'-oxohexyl)-indole, and 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole.

39. A composition according to Claims 34, 35, 36, 37 or 38 wherein said effective amount of active material is sufficient to kill fungi on agricultural crops and botanical species.

40. A composition according to Claims 34, 35, 36, 37 or 38 wherein said effective amount of active material is sufficient to kill fungi on agricultural livestock or animals, humans, other animals or fish.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/08216

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93D, 405; 435/252.1, 261; 534/573; 544/3; 562/443; 548/457; 549/29

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Merck Index

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	Journal of General Microbiology, Volume 128, issued 1982, Akhurst, "Antibiotic Activity of *Xenorhabdus spp.*, Bacteria Symbiotically Associated with Insect Pathogenic Nematodes of the Families Heterorhabditidae and Steinernematidae", pages 3061-3065, see entire document.	1, 2, 4, 7, 14, 16, 19, 21, 22, 24-28 ------- 3, 5, 6, 8-13, 15, 17, 18, 20, 23
X -- Y	R. GAUGLER et al, "Entomopathogenic Nematodes in Biological Control" published 1990 by CRC Printers (Florida), pages 271-284, see entire document.	1, 3, 4, 9, 11, 21, 22, 24, 25, 29-40 ------- 2, 5-8, 10, 12-20, 23, 26-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 OCTOBER 1994	NOV 10 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARIA OSOTEO Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/08216

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US, A, 4,837,222 (GREGSON ET AL) 06 June 1989, see entire document.	1, 3, 4, 9, 11 ------ 2, 5-8, 10
X -- Y	US, A, 4,672,130 (RHODES ET AL) 09 June 1987, see entire document.	1, 3, 4, 9, 11 ------ 2, 5-8, 10
X -- Y	FEMS Microbiology Ecology, Volume 53, issued 1988, Bleakley et al, "Characterization of primary and secondary forms of *Xenorhabdus luminescens* strain Hm", pages 241-250, see entire document.	1, 3, 4, 9, 10, 11, 21, 22, 24 ------ 2, 5-8, 12, 23
X -- Y	Applied and Environmental Microbiology, Volume 54, Number 6, issued June 1988, Richardson et al, "Identification of an Anthraquinone Pigment and a Hydroxystilbene Antibiotic from *Xenorhabdus luminescens*", pages 1602-1605, see entire document.	29, 30, 32, 33 ------ 31
X	Journal of Chemical Ecology, Volume 7, Number 3, issued 1981, Paul et al, "Antibiotics in Microbial Ecology", pages 591-597, see entire document.	29-33
X	Journal of General Microbiology, Volume 139, issued 1993, Sundar et al, "Antimicrobial activity and biosynthesis of indole antibiotics produce by *Xenorhabdus nematophilus*", pages 3139-3148, see entire document.	34-40
Y	Ann. Rev. Phytopathol., Volume 19, issued 1981, Hart, "Role of Phytostilbenes in Decay and Disease Resistance", pages 437-458, see entire document.	29-33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/08216

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A01N 25/00, 63/00; A61K 37/00; C12N 1/02, 1/12, 1/20; C07C 245/00; C09B 7/02

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/93D, 405; 435/252.1, 261; 534/573; 544/3; 562/443; 548/457; 549/29